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Substitution of Arginine for Glycine at Position 154 of the α1 Chain of Type I Collagen in a Variant of Osteogenesis Imperfecta: Comparison to Previous Cases With the Same Mutation

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A substitution of arginine for glycine at amino acid position 154 of the α1(I) collagen chain was found in a father and his three children. The phenotype of the patients includes manifestations of types I and III/IV osteogenesis imperfecta, but appears to be milder than that of the previously described two unrelated patients that had the identical mutation in the α1(I) collagen chain. The variability in the phenotype raises the possibility of epistatic loci or environmental effects on expression of the disorder.

KEY WORDS: phenotype variability, direct sequencing of PCR products, single base mutation

INTRODUCTION

Osteogenesis imperfecta (OI) is a heterogeneous group of connective tissue disorders whose hallmark is fragile bones. Severity varies from perinatal death to mild deformity of long bones with a history of fractures [Sillence et al., 1979; Sillence, 1981]. OI has been classified into four types based on clinical criteria [Sillence et al., 1979; Sillence, 1981]. The mildest OI is called type I and most patients have blue sclerae, a history of fractures and mild long bone deformity, that is in part responsible for short stature. Most of the patients have a height that falls between 2 and 3 standard deviations below that of controls; however, some are much shorter [Sillence et al., 1979; Sillence, 1981]. Perinatally lethal variants of OI are called type II, whereas moderate to severe variants are classified as types III and IV, with type III being the more severe and leading to progressive deformity of long bones, spine and skull [Sillence et al., 1979; Sillence, 1981].

Despite the clinical variability, all of the about 100 mutations causing OI that have been characterized to date were mutations in one of the genes for type I collagen [Kuivaniemi et al., 1991; Prockop, 1992; Prockop et al., 1994]. Type I collagen is a fibrillar collagen composed of two α1 chains and one α2 chain and characterized by a long triple-helical domain that is composed of a repetitive Gly-X-Y tripeptide structure [Prockop, 1990, 1992]. Glycine, the smallest amino acid, occurs in every third position and occupies the center of the triple helix where the three polypeptides are in closest proximity. The structure does not tolerate substitution of larger amino acids for glycine. Since there are 338 Gly-X-Y triplets in the triple-helical domain of type I collagen, there are some 338 critical amino acids in each chain that, when mutated, can potentially cause the disease. To date, the most N-terminal substitution of another amino acid for an obligatory glycine was cysteine for α1Gly19 [Kuivaniemi et al., 1991; Prockop et al., 1994].

The clinical variability of OI has been matched by extensive heterogeneity in the mutations causing the disease. Of the over 100 mutations characterized in unrelated individuals, about 75 are substitutions of other amino acids for obligatory glycines in either the gene for proα1(I) collagen or proα2(I) collagen [Prockop et al., 1994]. To date, most mutations have substituted other amino acids for glycines at different positions. At only 15 positions have glycines been substituted more than once by another amino acid. Of these, glycines at nine positions have been substituted by the same amino acid.

Here we describe a mutation that substitutes arginine for glycine 154 of the α1(I) chain in a family with OI. The identical mutation was previously character-

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ized in two unrelated individuals [Pruchno et al., 1991]. The phenotype in the family described here appears to be milder than that of the previously described patients. The variability of expression of identical mutations between this family and the previously described patients raises the possibility of epistatic loci or environmental effects on expression of the disease.

MATERIALS AND METHODS

Clinical Summary

All three children of a man with OI inherited the disorder. The parents were from El Salvador. The father had fractures and deformities of the lower limbs at birth. Although he sustained 16 fractures postnatally, mostly of the lower limbs, he did not sustain any additional fractures after puberty at age 16 (Table I). At age 32 years he weighed 39.9 kg and height was 116.9 cm (<3rd centile). He had blue sclerae, scoliosis, lower limbs deformed by bowing of the femora, dentinogenesis imperfecta, and moderate hearing loss. He was very active, walking independently of any braces, crutches or other support, and worked long hours in a factory treating furs. All three of his children (Fig. 1) had prenatal and perinatal fractures, low birth weight and length, blue sclerae and angulation deformities of the lower limbs. They all sustained multiple fractures during the first year(s) of life and had dentinogenesis imperfecta. The diagnosis of OI was made based on radiological evidence that documented cortical thinning with general osteopenia, bone deformities and signs of healed fractures.

Patient Material

Skin biopsies obtained from the third child and a normal control individual were used to establish skin fibroblast cultures. Blood samples were obtained from the other relatives. The study was approved by the Institutional Review Board and all samples were collected after informed consent. Total RNA was isolated from cultured skin fibroblasts using a procedure involving extraction with guanidinium isothiocyanate [Maniatis et al., 1982]. Genomic DNA was isolated from cultured skin fibroblasts or blood [Maniatis et al., 1982].

Analysis of Collagenous Proteins

Proteins were labeled by incubating the fibroblasts for 4 h in fresh Dulbecco's modified Eagle medium containing 50 μg/ml ascorbate and 50 μCi/ml L-(2,3,4,5-^{3}H) proline (100 Ci/mmol, Amersham Corp., Arlington Heights, IL) [Williams and Prockop, 1983; Bonadio and Byers, 1985]. The cell layer and media samples were harvested and processed separately. The newly synthesized proteins were digested with pepsin overnight at 4°C and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [Williams and Prockop, 1983; Bonadio and Byers, 1985].

Sequencing of PCR Products

About 50 μg of total RNA was used to synthesize single-stranded cDNA with MMVL reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). The single-stranded cDNA was used as template for polymerase chain reaction (PCR) with Taq DNA polymerase (Perkin-Elmer-Cetus, Emeryville, CA). Direct sequencing of PCR products was carried out as described [Spotila et al., 1991, 1994]. In brief, seven sets of α1 primers and six sets of α2 primers were used in PCR to generate overlapping products that included all the coding sequences of the proα1 chain and proα2 chain. Two rounds of asymmetric amplification were carried out to generate single-stranded DNA fragments. The PCR products were purified using the glassmilk method (Geneclean II, Bio101, La Jolla, CA) with a Biomek™ 1000 workstation (Beckman, Fullerton, CA). The purified PCR products were sequenced directly using Bst DNA polymerase [Earley et al., 1993] and an adapted Zymark Robot [Earley et al., 1994]. A total of

TABLE I. Clinical Findings in Patients With the α1Gly154 to Arg Mutation

	Reported previously[a]		Described here			
	I	II	III-A	III-B	III-C	III-D
Age at ascertainment (yrs)	35	26	32	2	1	1.2
Gender	M	M	M	F	M	F
Body height						
cm	109	96.5	116.9	76.5	65	68
centile	<3	<3	<3	<3	<3	<3
Body weight						
kg			39.9	9.4	6.4	7.1
centile	<3	<3	<3	<3	<3	<3
Fractures						
total number	60+	60+	16	3	5+	5+
since puberty	N	Y	N			
Needs crutches or wheelchair	Y	Y/N[b]	N	N	N	N
Active ambulation	N	N	Y	Y	Y	Y
Sclerae	Slightly blue	Light grey	Blue	Blue	Blue	Blue
Hearing loss	N		Y			

[a]See Pruchno et al. [1991].
[b]Reported as walking independently but also as using crutches.

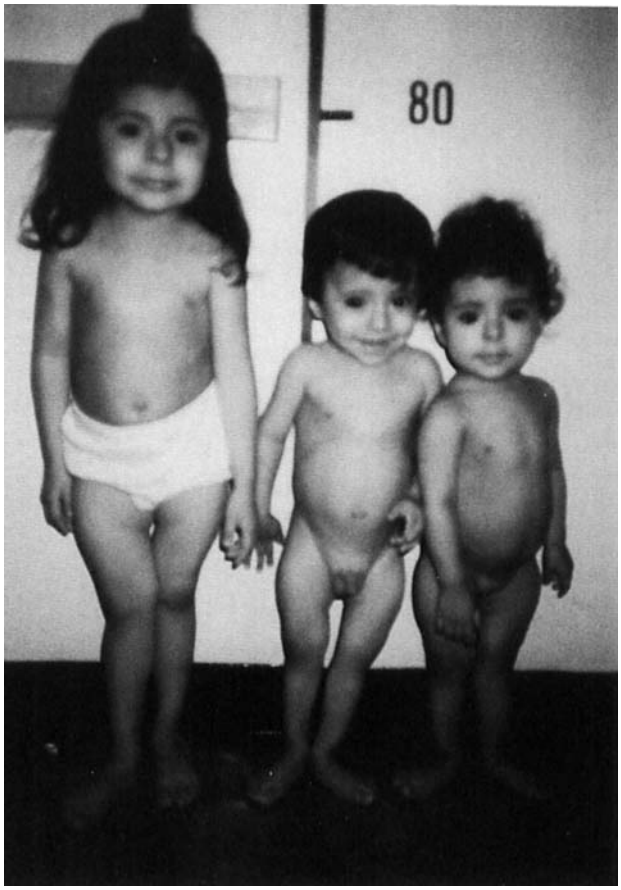

Fig. 1. Photograph of the three affected children at the ages of 4, 2 and 1½ yrs, respectively. There are signs of blue sclerae, bowing of the lower limbs, deformity of the chest in all three affected children. In the oldest child (**left**), the lower left limb is shorter than the right. The youngest child (**right**) was the propositus.

25 sequencing primers for α1 and 28 sequencing primers for α2 were used to sequence the respective cDNAs from each cell line. Seven or eight cell lines were processed in parallel. The sequences of the primers used to detect the mutation are shown in Table II.

Analysis of Point Mutation and Polymorphisms

All base changes that were detected during sequencing of the cDNA were confirmed by using genomic DNA as template in the PCR and digesting the genomic DNA PCR products with the appropriate restriction endonucleases. The polymorphic variant at nt 3306 corresponding to amino acid 897 of the α1 chain [Sokolov et al., 1991] was detected using AgeI which cuts the rarer allele. The polymorphic variant at nt 1529 corresponding to amino acid 392 of the α2 chain was detected with PvuII as described [Constantinou et al., 1991].

To confirm the mutation at nt 1077 corresponding to amino acid 154 of the α1 chain, primers Pr5 and Pr6, shown in Table II, were used to amplify a part of the gene containing the mutation. The mutation abolished a site for ApaI in the PCR product.

For quantitative analysis of the PCR products from the normal and mutant alleles, a nested PCR was carried out: the first PCR was performed with primers Pr7 and Pr8 (Table II) and the second PCR with primers Pr5 and Pr6 in which Pr5 was radioactively labeled and the PCR carried out for only 15 or 20 cycles. The PCR products were digested with ApaI, the fragments separated on 6% DNA sequencing gel (Sequagel-6, National Diagnostics, Atlanta, GA) and the volumetric quantitation carried out using Phosphorlmager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Biochemical Findings of the Type I Collagen Proteins

The pepsin-treated type I procollagens isolated from the cell layer and media of the patient's cultured fibroblasts were post-translationally overmodified in that the α chains migrated more slowly on SDS-PAGE than did the α chains from control fibroblasts (not shown). The slower migration was abolished by addition of α,α′ dipyridyl, an inhibitor of prolyl and lysyl hydroxylases, to the labeling medium (not shown).

Direct Sequencing of PCR Products

In order to define the mutation that caused the disease, we sequenced all the coding sequences of the cDNAs for proα1 and proα2 collagen. After amplification, PCR products were purified using a Biomek 1000 workstation and sequenced by a modified Zymark robot [Earley et al., 1994]. Seven or eight samples from different patients were loaded onto sequencing gels such that all reactions terminated with the same dideoxynucleotide (e.g., ddG) were adjacent to each other. A mu-

TABLE II. Primers Used to Detect and Confirm α1Gly154 Mutation

Primer number	Primer sequence	Location	Direction
Used to detect mutation:			
Pr1	AGGAAACTTTGCTCCCCAGC	Exons 5 and 6	Sense
Pr2	CGAGCTCCTCGCTTTCCTTCCTCT	Exon 21	Antisense
Pr3	GGTTCACCGCTGTTACCCTTGG	Exons 19 and 20	Antisense
Pr4	TCAGTGGTTTGGATGGT	Exon 12	Sense
Used to confirm mutation:			
Pr5	CCAGTGCTCAGTGGACTTA	Intron 14	Sense
Pr6	AAGTCACACCTGGGACAGA	Intron 15	Antisense
Pr7	GGGAACAAGGCTGTCTCCCATCTCAT	Intron 13	Sense
Pr8	GGAAGAGATGGCAGCTGCAAGTCACA	Intron 15	Antisense

tated base or polymorphism is detected by the presence of a band in a position where few or no other cell lines have a band (Fig. 2). For heterozygous individuals the new band comigrates with the normal band. The patient's cDNAs contained three single-base changes. The first change was a polymorphism at nt 1529 of the proα2(I) cDNA that converted the codon for amino acid 392 from -CCA- to -CCC- synonymous codons for proline [Kuivaniemi et al., 1988; Constantinou et al., 1991]. The second change was a polymorphism at nt 3306 of the α1(I) cDNA that converted the codon for amino acid 897 from -GCC- for alanine to -ACC- for threonine [Sokolov et al., 1991]. The third change converted -G- to -A- at nt 1077 and converted the codon for amino acid 154 of the triple-helical domain of the proα1 cDNA from -GGG-, a codon for glycine to -AGG- a codon for arginine.

Confirmation of the Mutation and Polymorphisms with Genomic DNA

The -G- to -A- mutation was located in exon 15 of the proα1(I) collagen gene [D'Alessio et al., 1988; Tromp et al., 1988] and eliminated a restriction site for ApaI (GGGCCC). To confirm the mutation, a pair of primers located in intron 14 and on the exon 15-intron 15 boundary of the gene for proα1(I) collagen, were used in PCR with genomic DNA from all members of the family and a normal control as templates. The products were purified and digested with ApaI. Samples from the control and the normal mother were digested into two bands; however, samples from the affected father and all three affected sibs yielded digestion patterns in which about half of the fragments were resistant to the restriction endonuclease (Fig. 3). To better estimate the ratio of mutant to normal allele, an additional pair of primers was used in a nested PCR, and one of the primers was radioactively labeled, and the PCRs were carried out for only 15 or 20 cycles. Three separate DNA isolations from the father were each used in two PCRs, and the results on these six data points gave a mutant to normal ratio of 1.250 with standard deviation of 0.106 (range 1.111 to 1.399; not shown). Two data points on the propositus gave values of 1.135 and 1.391 (not shown). The results in the father and the propositus were, therefore, indistinguishable from each other. The polymorphisms at amino acid nt 3306 corresponding to 897 of the α1 chain [Sokolov et al., 1991] and nt 1529, corresponding to amino acid position 392 of α2 chain [Kuivaniemi et al., 1988; Constantinou et al., 1991] were also confirmed by restriction digestions using genomic DNA as template in the PCRs.

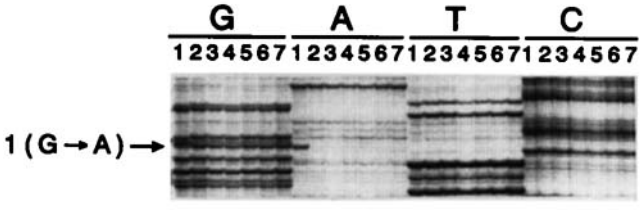

Fig. 2. Nucleotide sequence of the region from bp 1067 to 1094 in the α1(I) chain of type I procollagen from 7 OI patients. Direct sequencing of PCR product was performed with the Zymark Robot. Seven samples from different patients were loaded onto the sequencing gel such that all reactions terminated with the same dideoxynucleotide (e.g., ddG) were adjacent to each other. The arrow points to the position where both a G and an A comigrated in sample 1 (the propositus in this paper). The codon GGG for Gly^{154} was converted to AGG, a codon for arginine in the mutant allele.

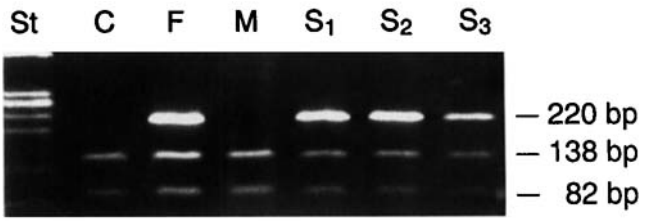

Fig. 3. Restriction digestion of PCR products with genomic DNA as a template. The PCR products were digested with restriction enzyme ApaI (GGGCCC). Samples from the control individual and the normal mother were digested into two bands. However, about half of the samples from the affected father and all three affected sibs were resistant to the restriction endonuclease. St, ΦxHaeIII digest as a molecular size marker; C, control sample; F, sample from father; M, sample from mother; S_1, S_2, S_3, samples from three siblings (S_3 is propositus).

DISCUSSION

The mutation presented here is identical to that described previously in two other unrelated patients [Pruchno et al., 1991; see I and II in Table I]. Interestingly, the phenotype of the affected members of the family described here is less severe than that of the previously reported cases. The father (III-A, Table I) of the family was ascertained through the propositus (III-D) and was about the same age at evaluation as the other two cases. He had many fewer fractures than the previously described patients (Table I) and lived an essentially normal life in that he walked without crutches and worked long hours in a factory treating furs. The diagnosis for the previous two cases was reported to be type III OI [Starman et al., 1989]. Four hypotheses to explain the different phenotypes are possible.

First, additional changes present in the genes for type I collagen exacerbate or ameliorate the effect of the disease-causing mutation. We sequenced the coding sequences for both alleles of proα1(I) and both alleles of proα2(I) collagen of the proband (III-D in Table I). Two changes were found in addition to the α1 Gly154 to Arg mutation, one in the cDNA for the α1 chain and the other in the cDNA for the α2 chain, but as explained in the Results, they were unlikely to alter the properties of the collagen.

A second source of variability in expression of the phenotype may be environmental or epigenetic effects. In terms of the OI phenotype, these include lifestyle and stochastic events that place stress on bones, the degree of medical treatment sought for fractures, and the duration and degree of immobilization. Such epigenetic factors may explain differences in number, location and severity of fractures but do not explain other differences in phenotype such as the color of sclerae and hearing loss. The expression of the genetic defect in the three sibs may also be less severe due to aggressive medicinal and surgical treatment regimens. The three sibs were treated with synthetic salmon calcitonin for

several years which led to improvement of bone density and diminished fractures [Castells et al., 1979]. Administration of synthetic salmon calcitonin to other patients with osteogenesis imperfecta has also been reported to increase bone density and resistance to trauma [Nishi et al., 1992].

Third, the phenotype in the father could have been mild because he was mosaic for the mutation in his somatic tissues. Since OI and other connective tissue diseases such as EDS IV are severe disorders and in many cases affected individuals do not give rise to offspring, a large number of the cases studied have been due to spontaneous mutations [Kuivaniemi et al., 1991]. In several instances germline mosaicism has been deduced for an apparently normal parent with more than one affected offspring [Cohn and Byers, 1990]. However, in only a few instances has mosaicism been demonstrated directly by examination of mutations in single cells or samples from several tissues [Cohn et al., 1992; Constantinou et al., 1990; Wallis et al., 1990; Kontusaari et al., 1992; Richards et al., 1992; Milewicz et al., 1993]. Such studies demonstrated that the mutated allele was present in from 0% to 100% of the cells in a particular sample. The signs and symptoms of an individual mosaic for a mutation may vary from none to pronounced, depending on when the mutation arose, and how it was distributed during development. Therefore, it is possible that the father in the family described here had a mild phenotype because the mutation occurred during his development and he was mosaic for it. We were unable to confirm or exclude the possibility since we were unable to obtain the necessary samples from various tissues, but our data on the quantitation of the mutant to normal allele ratio, when using DNA isolated from blood, do not support mosaicism (not shown).

Fourth, expression of phenotype may be altered by the influence of genes at completely different loci, called modifier genes. The combined effect of numerous modifying genes is frequently referred to as the genetic background effect. Bone formation involves a large number of molecules besides type I collagen [Heinegård et al., 1989]. These include proteoglycans, bone morphogenetic proteins, osteopontin, osteocalcin, bone sialoprotein and other collagens, some of which may be common to bone and sclerae. Linkage studies have indicated that in some families with OI, the genes for type I collagen are either not linked or excluded [Aitchison et al., 1988; Wallis et al., 1993], supporting the role of other genes in causing or modifying OI. Recent sib-pair analyses have shown that the number of copies of an allelic variant of the vitamin D receptor correlates with low bone density [Morrison et al., 1994]. A frequent or common genetic predisposition to low bone density such as the vitamin D receptor variant provides a plausible explanation for a more severe phenotype in some individuals with OI due to identical mutations in the genes for type I collagen. However, the vitamin D receptor variants are unlikely to influence the color of the sclerae but illustrate a plausible role for modifying genes in the expression of OI phenotype. In addition, recent experiments with a line of mice that are transgenic for a minigene for COL1A1 that causes an OI-like phenotype suggest that variable expression of phenotype may be an inherent property of some genotypes [Khillan et al., 1991; Pereira et al., 1994]. The inbred transgenic mice have a uniform genetic background and genotype with respect to the transgene, yet the phenotype varied from mild to severe in terms of the number and nature of fractures documented.

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